Journal of the American Mosquito Control Association, 24(4):508–512, 2008

A QUANTITATIVE IN VITRO ASSAY FOR CHEMICAL MOSQUITO-DETERRENT ACTIVITY WITHOUT HUMAN BLOOD CELLS[1]

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ABSTRACT. We report that an aqueous solution containing 10^{-3} M adenosine triphosphate (ATP) and citrate-phosphate-dextrose-adenine (CPDA-1) can effectively replace transfusable human red blood cells in an in vitro Klun and Debboun bioassay system for evaluating chemicals for mosquito feeding-deterrent activity, using either *Aedes aegypti* or *Anopheles stephensi*. These species fed with similar avidity through collagen membrane covering aqueous 10^{-3} M ATP plus CPDA-1 or red blood cells in CPDA-1 supplemented with ATP. In a 2nd experiment, we evaluated the feeding-deterrent activity of N,N-diethyl-3-methylbenzamide and a newly discovered natural product chemical, (-)-isolongifolenone, against these 2 mosquito species. We found that the feeding-deterrent efficacy of the 2 chemicals was similar whether the feeding stimulant was red blood cells supplemented with ATP or ATP alone with CPDA-1. Since the use of human red blood cells in bioassays raises important health and logistic issues, aqueous ATP with CPDA-1 is a reasonable alternative to human blood cells for routine in vitro chemical screening.

KEY WORDS *Aedes aegypti*, *Anopheles stephensi*, N,N-diethyl-3-methylbenzamide, ATP, mosquito biting, isolongifolenone, CPDA-1

INTRODUCTION

The in vitro Klun and Debboun (K&D) bioassay system, for use in high-throughput screening of chemicals to identify new compounds having potential use as topical mosquito deterrents on humans (Klun et al. 2005), has proved fruitful in chemical screening. Its use led to the discovery and patents of 2 new repellent compounds, callicarpenal (Cantrell et al. 2005, Carroll et al. 2007) and (-)-isolongifolenone (Zhang et al. 2008). An important component of this system is a water-bath-warmed (38°C) bloodfeeding reservoir containing 6 adjacent wells, each holding 6 ml of outdated transfusable packed human red blood cells supplemented with adenosine triphosphate (ATP). The blood-filled wells of the reservoir are covered with a collagen membrane, and the assembled device serves as a pseudo-host for feeding by *Aedes aegypti* (L.) or *Anopheles stephensi* Liston in a 6-celled K&D module (with 5 females/cell).

The use of human red blood cells in the bioassays is problematic because (1) expired packed red blood cells are not always available from the local blood bank, and (2) strict human-blood-use protocols must be followed because of blood-borne pathogen risks. These risks, although low, require special laboratory biosafety training for all personnel handling the human blood cells (USDHH et al. 2007). These shortcomings provided the impetus for discovery of an alternative to the human blood cells used to stimulate mosquito feeding in the bioassay.

To understand the connection between the K&D bioassay system and our testing of mosquitoes using an aqueous solution containing ATP, we need to explain how human blood is typically handled prior to testing. Whole human blood is separated into blood components by a standardized process prescribed by the American Association of Blood Banks. The process involves isolation of red blood cells by centrifugation of whole blood, removal of plasma, and suspension of the cells in citrate-phosphate-dextrose-adenine (CPDA-1), an anticoagulant preservative (AABB 2005). We obtained the red blood cell preparations from Blood Services, Department of Pathology and Areas Laboratory Services, Walter Reed Army Medical Center, Washington, DC.

Cells refrigerated in CPDA-1 have a shelf life of about 5 wk, and after that the cells are no longer considered useful for transfusion. We found that *Ae. aegypti* readily bit and engorged on red blood cell preparations offered to the mosquitoes in the K&D reservoir up to 1 month after cell expiration date without an ATP supplement. However, cells stored longer would stimulate biting and engorgement only if they were fortified with ATP. In other preliminary tests, we also found that CPDA-1 alone would not stimulate mosquito feeding. Thus, we won-

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2008		**00-00-2008 to 00-00-2008**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
A Quantitative in Vitro Assay for Chemical Mosquito-Deterrent Activity Without Human Blood Cells	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Army Medical Department center and School,Academy of Health Sciences,Department of preventive Health Services,Fort Sam Houston,TX,78234**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
see report

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**5**	

dered whether a solution with ATP but without red blood cells would stimulate feeding.

The literature in this area suggested that this might be the case. Stimulation of feeding response in *Ae. aegypti* and other bloodfeeding insect species by ATP was first described by Galun et al. (1963), and the phenomenon was studied extensively by Galun and others (Friend 1978, Smith and Friend 1982). In an electrophysiological study with *Ae. aegypti*, Werner-Reiss et al. (1999) found that labral apical sensilla signaled the presence of adenine nucleotides but did not distinguish ATP concentrations differing by orders of magnitude. Galun et al. (1985), however, showed that *Ae. aegypti* engorging behavior was concentration dependent over a range of 10^{-3} M to 10^{-6} M ATP. In living *Xenopus* oocytes, the physiological sub-membrane concentration of ATP is approximately 10^{-3} M ATP (Gribble et al. 2000), the concentration we used.

Two experiments were conducted in the K&D bioassay system, each using *Ae. aegypti* and *An. stephensi* adult females. Experiment 1 evaluated the comparative feeding stimulation effects of red blood cells in CPDA-1 (about 1 month past blood bank expiration date) supplemented with ATP (blood-ATP) compared with ATP alone in CPDA-1 (ATP-CPDA). We also looked at the statistical power we had to find differences between these 2 feeding stimulants. Experiment 2 evaluated the feeding-deterrent effects of N,N-diethyl-3-methylbenzamide (deet) and a newly discovered deterrent natural product, (-)-isolongifolenone, using each of the 2 feeding stimulants.

MATERIALS AND METHODS

Insects

Female *Ae. aegypti* (red eye Liverpool strain) and *An. stephensi* (Delhi strain, traced by Shute and Maryon [1960] to a colony established in 1947) were from colonies maintained at the Walter Reed Army Institute of Research, Silver Spring, MD. The insects were reared (Gerberg et al. 1994) by feeding larvae ground tropical fish flakes (Tetramin Tropical Fish Flakes; Tetra Sales, Blacksburg, VA). Adult mosquitoes were held at 12:12 (L:D)-h photoperiod at 27°C and 80% RH with cotton pad moistened with 10% aqueous sucrose solution. Mated nulliparous *Ae. aegypti* and *An. stephensi* females (5–10 days old) were used in the testing. *Aedes aegypti* had neither sugar nor water 24 h before testing, and *An. stephensi* had access only to water in the pretesting 24-h period. From previous experience, we knew that feeding by *An. stephensi* in tests could be maximized by allowing them drinking-water access before testing and by minimizing the length of time they were held in the K&D modules prior to testing. The time-related optimization was accomplished by first preparing the reservoirs for testing, and then collecting, transporting, and initiating testing of the mosquitoes in the K&D modules as rapidly as possible (approximately a 5-min interval between preparation of the reservoirs and start of testing). In contrast, we knew from experience that a short pretest-residence time in the K&D module and having water pretest was not critical with *Ae. aegypti*. These mosquitoes were routinely transferred to the K&D modules 1–2 h before being used in a test.

Chemicals

The CPDA-1 (AABB 2005) was prepared by dissolving 33.3 g sodium citrate, 0.376 g monobasic sodium phosphate (Fisher Scientific Chemical Co., Fairlawn, NJ), 4.02 g dextrose, and 0.035 g adenine (Aldrich Chemical Co., Milwaukee, WI) in 63 ml water. The ATP (Aldrich Chemical Co.) was added to the CPDA-1 or red blood cells in CPDA-1 to yield 10^{-3} M ATP. All ATP-containing preparations were freshly prepared on the day of their use. Deet was obtained from Morflex, Inc. (Greensboro, NC) and (-)-isolongifolenone was synthesized in our laboratory as described by Zhang et al. (2008). The compounds were >98% pure chemically according to gas chromatography analyses.

Bioassays

All bioassays were conducted with the in vitro K&D system positioned in a PURAIR ductless chemical fume hood (Air Science USA LLC, Fort Myers, FL), and tests were conducted at 24–26°C and 24–51% RH in ambient laboratory light over several days. The upper surface of each water-bath-warmed reservoir was coated with a thin layer of high-vacuum silicone grease (Dow Corning Corp., Midland, MI), and the 6 reservoir wells were filled with either blood-ATP or ATP-CPDA. The full cells were covered with a 29.7 × 7.1-cm piece of Edicol collagen membrane (Devro, Sandy Run, NC). A strip of organdy cloth (G Street Fabrics, Rockville, MD) of the same dimensions, either chemically treated or untreated, was then placed over the membrane and a Teflon separator was positioned atop the cloth (the separator prevented contact of the K&D module with the chemically treated cloth). Mosquitoes contained in the 6 cells of the K&D module were placed over the Teflon separator for exposure to treatments. In Experiment 1, 12 wells of 2 water-bath-warmed reservoirs positioned in series were alternately filled with blood-ATP and ATP-CPDA. Trap doors of the 2 6-celled K&D modules, each containing 5 mosquitoes, were opened to expose the mosquitoes to the respective treatments for 3 min, and the number biting (proboscis inserted through untreated organdy

cloth and collagen membrane plus those that had engorged and flew from the feeding surface were recorded for each cell). A replicate consisted of a cell of 5 mosquitoes offered blood-ATP and an adjacent cell of 5 mosquitoes offered ATP-CPDA. A total of 200 *Ae. aegypti* and 200 *An. stephensi* mosquitoes were tested against each treatment (40 replicates/treatment).

In Experiment 2, deet and (-)-isolongifolenone in 95% ethanol solutions (4.545 nmol/μl) were each randomly applied to 2 of 6 3 × 4-cm rectangular cloth areas marked with ink pen on a 29.7 × 71-cm piece of organdy cloth, and 2 cloth areas were treated with ethanol alone as control. We uniformly applied ethanol solutions (110 μl) to the rectangular cloth areas. The solutions were always applied 0.5 cm beyond the ink-marked areas to ensure that mosquitoes would be exposed only to chemically treated cloth. The air-dried treated cloth (25-nmol-compound/cm^2 cloth) was placed over the membrane-covered wells filled with either blood-ATP or ATP-CPDA. The 25-nmol-compound/cm^2 dose was used here because previous bioassays with deet at this dose consistently showed 60–80% suppression of mosquito biting compared to controls (Klun et al. 2003). As before, the Teflon separator was placed over the treated cloth to prevent contact of the K&D module with the chemically treated cloth.

A replicate consisted of 6 randomized cloth-treatment areas covering the 6 wells of a reservoir with 2 of deet, 2 of (-)-isolongifolenone, and 2 of control ethanol-treated cloth areas, each exposed to 5 mosquitoes positioned over wells filled with the 2 different feeding-stimulant treatments. Only data sets in which 3 or more females fed in each control cell were analyzed, as lower feeding rates were indicative of substandard feeding readiness of that group of mosquitoes. We replicated tests over time, and each treatment was tested against 200 *Ae. aegypti* females (40 replicates) and 200 female *An. stephensi* (40 replicates), and we recorded the number of mosquitoes biting in each cell.

Statistical methods

Two analyses were conducted for Experiment 1. In the 1st analysis, we wanted to determine if there were different responses to the blood-ATP versus the ATP-CPDA treatment, find out if the 2 different species responded similarly, and test for an interaction between the 2 factors. For this analysis, we used a generalized linear model with a logit link (proportion biting versus proportion not biting) (McCullagh and Nelder 1989), using R software (R Development Core Team 2007). We first checked for overdispersion by estimating the dispersion parameter using a quasi-binomial model. Since this parameter was estimated to be near 1.0, we used the binomial model. The 2nd analysis investigated the power we had to determine differences in the 1st analysis using Monte Carlo simulation methods. Here we used simulated data with known (true) differences between proportions of biting mosquitoes and tallied the number of simulations where the proportions differed significantly, using the same R procedure we had used to analyze the original data.

A simulated data set was created by first drawing 200 random numbers from a uniform distribution on [0, 1], and assigning all those below a specified value (say, $p_1 = 0.7$) to ''biting mosquitoes,'' and all others to ''non-biting mosquitoes.'' This creates a sample from a binomial distribution with a known parameter p_1. Then a 2nd draw of another 200 random numbers from a uniform distribution on [0, 1] was treated similarly, except a different cutoff (say, $p_2 = 0.8$) was used. This simulated data set was analyzed as described above to see if the 2 groups were declared statistically different at $\alpha = 0.05$. We did 2,000 simulations for each pair of known proportions (p_1 and p_2), using various values of p_1 and p_2. We used these results to estimate the power to discriminate between each pair of known proportions (with a sample size of 200 mosquitoes per group) as the percentage of the simulations with a P-value <0.05. Each set of simulated data is analogous to testing 1 of the 2 species on the 2 feeding treatments.

In Experiment 2, all parts were analyzed using a generalized linear model with a logit link, as described above. The dispersion parameter for all experiments was first estimated using a quasi-binomial model. All were estimated to be near 1.0 (indicating no overdispersion), so data were assumed to follow a binomial distribution. Because the 3 treatments used were tested against each other for significant differences, we needed to make a multiple comparisons adjustment and used the Holm method (Holm 1979). Results were back-transformed from the logit scale to the original scale (proportion of nonbiting mosquitoes) for ease of interpretation. An approximate 95% confidence interval on the proportion was calculated by back-transforming the coefficient $\pm 2 \times$ SE (coefficient), where the coefficient is the estimated proportion of non-biting mosquitoes on the logit scale.

RESULTS

Experiment 1

We found no significant difference ($P = 0.313$) in the mosquitoes' responses to the blood-ATP versus the ATP-CPDA feeding-stimuli treatments (differences were about 5%; Table 1). There was a small but significant difference ($P = 0.033$) between the species biting rate, with *Ae. aegypti* biting about 10% more frequently than *An. stephensi*. There was no significant interaction effect ($P = 0.764$).

Table 1. Proportion of mosquitoes not biting in response to human red blood cells in citrate-phosphate-dextrose-adenine (CPDA-1) supplemented with adenosine triphosphate (ATP) or ATP alone in CPDA-1 with Klun and Debboun reservoir wells covered with collagen membrane.

Treatment	Proportion not biting
Ae. aegypti	
Blood cells	0.51
ATP	0.55
An. stephensi	
Blood cells	0.60
ATP	0.66

For proportions built from binomial data, their variances depend on the mean (largest when the proportion is 0.5), so detecting a difference of 0.10 between 2 proportions will have different power depending on whether the 2 proportions are, say 0.5 and 0.6, or 0.85 and 0.95 (the latter will have higher power). For our simulations, we found that we had at least 80% power to detect differences in proportions of 0.15, regardless of where (between 0 and 1) the 2 proportions lay. A difference of 0.10 with 80% power is not detectable using sample sizes of 200 until the smaller proportion approaches 0.80 (at biting rates of 0.80 and 0.90, or equivalently 0.20 and 0.10, power is about 93%). The biting rates in this experiment were nearer 0.50 (Table 1), so they occurred in a region where differences of <0.15 would not be considered significant for a sample size of 200. Note that, when testing the actual data, we found significance for a difference of 10%. Since the analysis was conducted in an ANOVA framework, the sample sizes used in the tests of main effects are larger ($n = 400$) because there is pooling over the 2 treatments when testing species differences. This power analysis was also useful for interpreting Experiment 2. Since mosquito biting rates in the presence of repellents were greatly reduced, the simulations show that under these circumstances we had the power to detect small differences between repellents (conversely, if differences were not declared significant, then we are confident mosquitoes responded similarly to the 2 repellents).

Experiment 2

Results presented in Table 2 were similar for both species of mosquitoes and both feeding stimuli. The 2 repellents significantly reduced the number of feeding mosquitoes ($P < 0.05$), and for *Ae. aegypti*, (-)-isolongifolenone had significantly ($P < 0.05$) fewer feeding mosquitoes than deet. At this concentration, *Ae. aegypti* showed a greater response to the repellents than *An. stephensi* (both fed at about the same proportion when no repellent was present, but a smaller proportion of *Ae. aegypti* fed in the presence of a deterrent). The proportion of feeding *Ae. aegypti* was similar for the 2 feeding stimuli for both compounds. However, with *An. stephensi* for (-)-isolongifolenone but not for deet, the proportion feeding on 10^{-3} M ATP-CPDA was almost 20% lower than for blood-ATP. Unfortunately, direct comparisons between feeding stimuli in this experiment cannot be made because the trials for each of the 2 feeding stimuli were run on different days. From our experiences with these mosquitoes, it is known that day-to-day variation can be considerable.

DISCUSSION

Our results show that feeding rates for feeding stimulants of either blood-ATP or ATP-CPDA are essentially the same for *Ae. aegypti* and *An. stephensi* mosquitoes. Further, decreases in feeding rates when mosquitoes sense repellent-treated cloth to feed is also similar for the 2 feeding stimulants, though *Ae. aegypti* seems more sensitive to the repellents than *An. stephensi*, consistent with other studies (Klun et al. 2003). Given sampling error and day-to-day variation, results from trials with blood-ATP and ATP-CPDA are sufficiently alike that substituting ATP-CPDA for the problematic blood-ATP

Table 2. Proportions of mosquitoes not biting with approximate 95% confidence intervals for in vitro Klun and Debboun module feeding-deterrent bioassays. Different letters denote significant differences between compounds within a species–stimulus combination.[1]

Treatment	Blood-ATP	ATP-CPDA
Mean proportions of 200 *Ae. aegypti* not biting		
Control	0.35 (0.29, 0.42)a	0.39 (0.32, 0.46)a
Deet	0.81 (0.75, 0.86)b	0.90 (0.83, 0.92)b
(-)-Isolongifolenone	0.93 (0.88, 0.95)c	0.94 (0.90, 0.97)c
Mean proportions of 200 *An. stephensi* not biting		
Control	0.35 (0.29, 0.42)a	0.36 (0.30, 0.43)a
Deet	0.64 (0.57, 0.71)b	0.60 (0.53, 0.67)b
(-)-Isolongifolenone	0.75 (0.68, 0.81)b	0.57 (0.50, 0.64)b

[1] Blood-ATP, red blood cells in citrate-phosphate-dextrose-adenine (CPDA-1) supplemented with adenosine triphosphate (ATP); ATP-CPDA, ATP alone in CPDA-1.

feeding stimulant should eliminate the difficulties of working with expired blood. Thus, in the K&D bioassay system use of human red blood cells can be discontinued without loss of assay power, at least for these 2 mosquito species, making it an efficient and convenient in vitro screening system. It is conceivable that in vitro bioassay systems other than the K&D system might also find that replacement of blood cells with ATP-CPDA useful. It should be noted, however, that although ATP-CPDA stimulates feeding and engorging by *Ae. aegypti* and *An. stephensi*, the chemicals are not a substitute for blood cells in mosquito egg production.

ACKNOWLEDGMENTS

We thank John C. Davis and Sandra Lara of the Agricultural Research Service for technical assistance in this project and Russell Coleman, Walter Reed Army Institute of Research, Division of Entomology, Silver Spring, MD, for making the mosquitoes available for the studies.

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